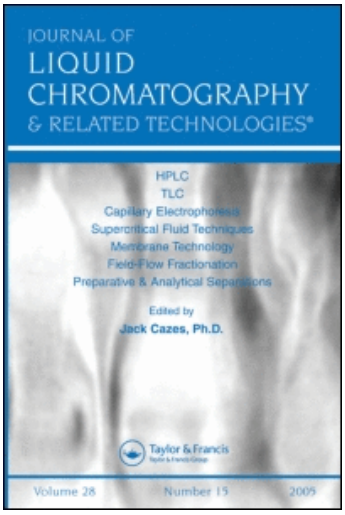

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PLANT HORMONE ANALYSIS BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Countercurrent chromatography (CCC) has been successfully applied for the separation of plant hormones; namely, indole auxins, gibberellins, cytokinins and abscisic acid. In our present study three different types of CCC devices were evaluated for their performance in separation of plant hormones with a special emphasis on analysis and purification of abscisic acid (ABA). A large-scale preparative CCC apparatus consisting of a slowly rotating coil assembly was used for preliminary separations of ABA from a large volume of crude plant extracts. The toroidal coil planet centrifuge (CPC) for analytical-scale separations was subsequently applied for purification of ABA, the final confirmation being obtained by HPLC and combined gas chromatographic-mass spectrometric method. This two-step procedure utilizing preparative CCC and toroidal CPC was successfully applied for determination of ABA content in several plant tissues. A recently introduced high-speed CCC apparatus was tested for semipreparative separation of ABA and indole-3-acetic acid. The method yielded high peak resolution within 2 hours.

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*Part of the experimental work described in this paper was performed at the Plant Hormone Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705, where the author was previously employed.

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INTRODUCTION

Several new innovations in the analytical methods, especially in chromatography, have greatly improved our separation capabilities recently that have helped to detect trace quantities in the parts-per-million (ppm) to parts-per-billion (ppb) range of several organic compounds. This is especially true for the separation and analysis of pesticides and plant growth substances. The chromatographic methods that received official and legal acceptance as the test methods include gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC) and a combined gas chromatography-mass spectrometry (GC-MS) which are capable of detecting trace (ppb) amounts in plant and animal tissues, foods and pharmaceuticals, besides pollutants from water and air. Furthermore, advances in the analytical methods for separation work are continuously sought for accurate and rapid determination in efficient and reliable manner either to complement or to supersede the existing techniques. A few such advances as coupling the liquid chromatography to mass spectrometer (LC-MS), one mass spectrometer to another mass spectrometer (MS-MS) and the GC and LC instruments to Fourier transform infrared spectrometers (GC-IR and LC-IR) which are further coupled to computers for total automation appear to be in the-developmental stages as a future generation of analytical instruments.

One recent development based on the principles of liquid chromatography and countercurrent distribution is known as the countercurrent chromatography (CCC) which has been introduced to separation science by Ito and his coworkers (1). They have been continuously refining the method for efficient separation of not only simple organic molecules but also high molecular weight polymers and cell particles. Several prototype instruments

were devised by Ito, and some of them are now commercially available (1,2). We have evaluated three instruments for their separation and analytical capabilities in the present work.

Several plant hormones and other growth substances appear to regulate the growth, development and reproduction of all higher plants (3). They are present at ppm to ppb levels in the plant tissue. They are required to study several metabolic changes including the stress-related phenomenon and the photosynthetic process, and also other biochemical and physiological implications during the plant growth and developmental process. We are especially interested in the analysis of ABA because of its involvement in the seed dormancy, stomatal closure and environmental stress besides its primary inhibitory function during the plant growth. Several analytical methods such as HPLC, GLC and GC-MS are now used for separation of plant hormones, and particularly for the analysis of ABA from plant tissues (3). As a continuation of our ongoing program for refinement and development of analytical methods for plant hormone analysis, we have examined the countercurrent chromatography for its potential applications in this field (4) and now report here our assessment of three CCC instruments for possible routine laboratory use.

MATERIALS AND METHODS

Apparatus

In the present study the following three types of CCC devices were evaluated for their performance. The general operating conditions of these instruments are summarized in TABLE 1.

TABLE 1.

General Operating Conditions for CCC Instruments in Plant Hormone Analysis

CCC Instrument	Flow rate (ml/hr)	Revolutional speed (rpm)
1. Toroidal coil planet centrifuge	2.5-4.0	450-500
2. Preparative CCC with rotating coil assembly	180-190	40-50
3. High-speed preparative CCC: small column	75-80	800-850
medium column	240	800

1) Toroidal Coil Planet Centrifuge (Toroidal CPC):

Figure 1 shows a simple table top model of the toroidal coil planet centrifuge (5,6) which is used for analytical-scale separations ranging from a few micrograms to milligram quantities. It has a rotatory frame driven by a motor around the stationary pipe mounted on the central axis of the centrifuge and holds a pair of symmetrically spaced cylindrical holders (10 cm from the central axis), one of which (15 cm O.D.) has a coiled column while the other holder (10 cm O.D.) carries a counterweight to balance the centrifuge system. Each holder is equipped with a plastic gear (Winfred N. Berg, Inc.) which is coupled to an identical sun gear mounted around the central stationary pipe. The gear arrangement produces the desired planetary motion of the holders, i.e., revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity in the same direction. For mechanical stability of the centrifuge, the free end (right side) of the rotatory frame is coaxially connected to a short coupling pipe, which is supported by a stationary wall member of the centrifuge through a ball bearing. The instrument employs a long column, typically 50 m of 0.55 mm I.D. PTFE tubing (Zeus Industrial Products, Raritan, N. J.) wound (8500 turns) on a 1.5 mm flexible core (13-m long)

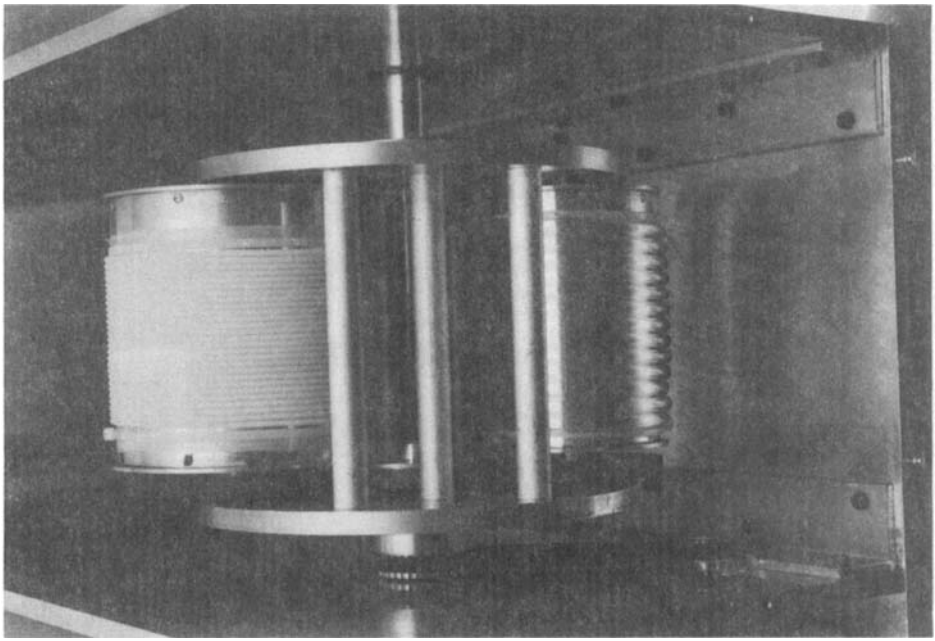

Figure 1. Toroidal coil planet centrifuge.

which is, in turn, coiled onto a (15 cm diameter) column holder. The gear drive provides a centrifugal force field that varies in intensity and direction as the column holder revolves around the central axis. The speed is adjustable up to 1000 rpm providing a maximum centrifugal force of about 450 xg. with a motomatic speed control unit (Electro-Craft).

The coiled column (total capacity: 18 ml) is first filled with a stationary phase of the pre-equilibrated two-phase solvent with the aid of either a Chromatronix Cheminert metering pump or a Milton-Roy pump. The test sample solution (50 µl) is typically introduced through a sample injection port and the mobile phase is pumped (pressure: 400-500 psi) through the column (2-4 ml/hr)

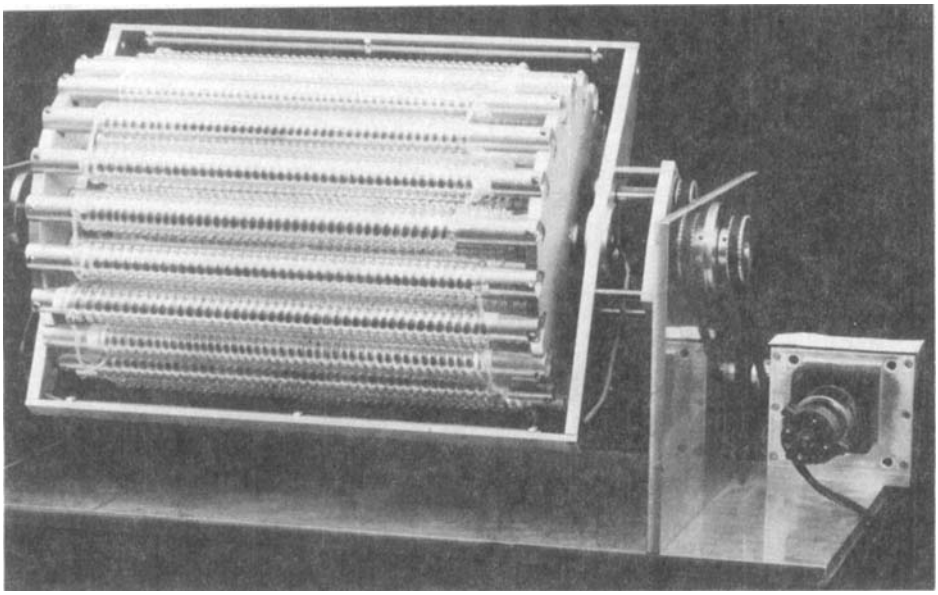

Figure 2. Preparative Countercurrent Chromatograph

while the apparatus is run at a desired revolutional speed (400-500 rpm). The column provides from 2000 to 6000 theoretical plates. The eluate is continuously monitored with either an LKB Uvicord III or an LKB Uvicord S at 206, 260 or 280 nm, depending on the test samples, and then collected with a fraction collector.

2) Preparative Countercurrent Chromatograph with Rotating Coil Assembly. A bench-top model of preparative CCC (FIG. 2) that performs efficient separations of gram-quantity samples was used to handle crude extracts of plant samples. The instrument design (2) consists of a coiled tube assembly that slowly rotates around its horizontal axis in a gravitational field. The stationary phase is retained by gravity in a large-diameter coil typically made of 50 turns of glass tubing with 0.5 cm

I.D., 2.5 cm helical diameter, and 90 ml capacity. A maximum of 30 columns can be mounted on the holder, 10 on the inner ring and 20 on the outer ring. The desired number of columns can be connected in series tail-to-head with PTFE tubing.
Although the rotational speed of the column assembly can be regulated up to 300 rpm, a maximum rate of 50-100 rpm is ideal for the glass column assembly. A large column (500 helical turns) consisting of 10 coils on a 2.5-cm diameter core connected in series has a capacity of approximately 900 ml. When 1 g sample (in 30 ml solvent) is introduced into the column, it is separated with a theoretical plate efficiency ranging from 800 to 1000 when the mobile phase is pumped at 120 ml/hr (revolution speed: 40-60 rpm depending on the stationary phase used).

3) High-speed Preparative Countercurrent Chromatograph.
This newly introduced CCC centrifuge performs fast and efficient separations in both analytical and preparative scales. It belongs to a member of the coil planet centrifuge which produces a synchonous planetary motion identical to that in the toroidal CPC. The design of the apparatus (FIG. 3) is also identical to that in the toroidal CPC except that the separation column consists of multiple layers of coiled PTFE tubing coaxially wound around a spool-shaped holder of 10 cm in diameter. In order to facilitate the preparation of this multi-layer coil column, the holder is made removable from the rotatory frame simply by loosening a pair of screws.

The unique feature of this CCC scheme is derived from an intriguing hydrodynamic motion of the two immiscible solvents in the multi-layer coil. Under the synchronous planetary motion the two solvent phases are subjected to a rapid countercurrent flow along the length of the coil, the upper phase traveling

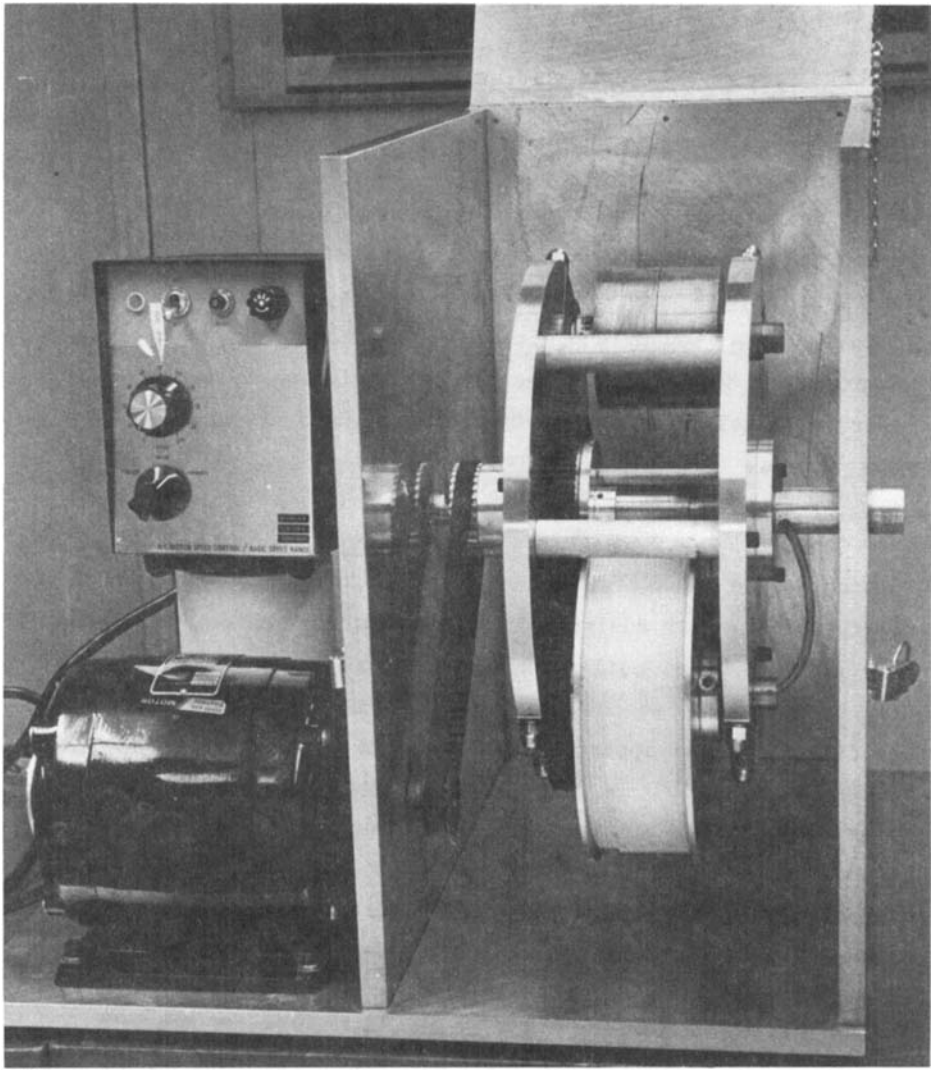

Figure 3. High-speed countercurrent chromatograph

toward the internal head-end and the lower phase, the external tail-end of the coil. This hydrodynamic motion establishes highly efficient partitioning of solutes with an excellent retention of the stationary phase under an unusually high flow rate of the mobile phase. Therefore, the method yields high peak resolution in a few hours of elution.

In the present study, separations are performed with two different I.D. columns - the medium size column with 130 m long. 1.6 mm I.D. and 285 ml capacity and the small size column with 170 m long, 1.0 mm I.D. and 140 ml capacity. The coil is first filled with the stationary phase followed by sample injection through the sample port. Then, the mobile phase is pumped into the column while the apparatus is run at 800 rpm. Both the sample solution and the mobile lower phase are introduced through the internal head-end of the coil. (If the mobile phase is the upper phase, they should be introduced through the external tail-end of the coil.) The flow rate applied to the medium column is 240 ml/hr and that to the small column, 80 ml/hr. The eluate is continuously monitored with an LKB Uvicord S at 260 nm and fractionated with an LKB fraction collector.

Sources of Hormone Samples

All of the test compounds were obtained from several commercial sources and used without further purification. Scarification of Zoysia grass seeds with alkali followed by extraction with diethyl ether-ethyl acetate (1:1) gave an extract which was acidified ($p_H 2$) with hydrochloric acid. The crude acidic extract was purified by HPLC on a reversed-phase μ Bondapak C_{18} column (Waters Associates) using methanol water (80:20) and the fraction corresponding to the retention volume of standard ABA sample was

collected and analyzed by CCC. Soybean seeds (400 g) were homogenized to a powder suspension with Polytron for 15 min with 700 ml of a mixture of methanol-water (80:20) and the homogenate was stored for 16 hr. After removing the insoluble material, the filtrate was concentrated to remove methanol. The solution was adjusted to p_H 2 and extracted with methylene chloride which was evaporated to give the acidic plant extract. The extract was then analyzed by CCC. Avocado fruit, after maceration, was extracted with 80% aqueous methanol (20:80) and kept at 4°C overnight. After removing the insoluble material, and the filtrate was concentrated. The aqueous extract was adjusted to p_H 2 with hydrochloric acid and extracted with diethyl ether (P.H. Terry unpublished procedure). Evaporation of solvent yielded an acidic extract which was subjected to CCC separation. Final confirmation for ABA was obtained by GC-MS analysis of the corresponding methyl ester.

RESULTS AND DISCUSSION

1) Toroidal CPC. Previously, we have reported the partition coefficients for indoles, gibberellins, cytokinins and ABA in different two-phase solvent systems. These systems were used for countercurrent chromatographic separation of plant hormones utilzing a toroidal coil planet centrifuge, referred here as the analytical CCC (4). We found that the naturally occurring indoles in the plant tissue can be separated in two-phase solvent systems, namely hexnane-ethyl acetate-methanol-water (0.6:1.4:1:1) and chloroform-acetic acid-water (2:2:1) because of their differences in partition coefficients ranging from 0.1-2.0. However, in the present study, we have chosen the latter solvent system, in

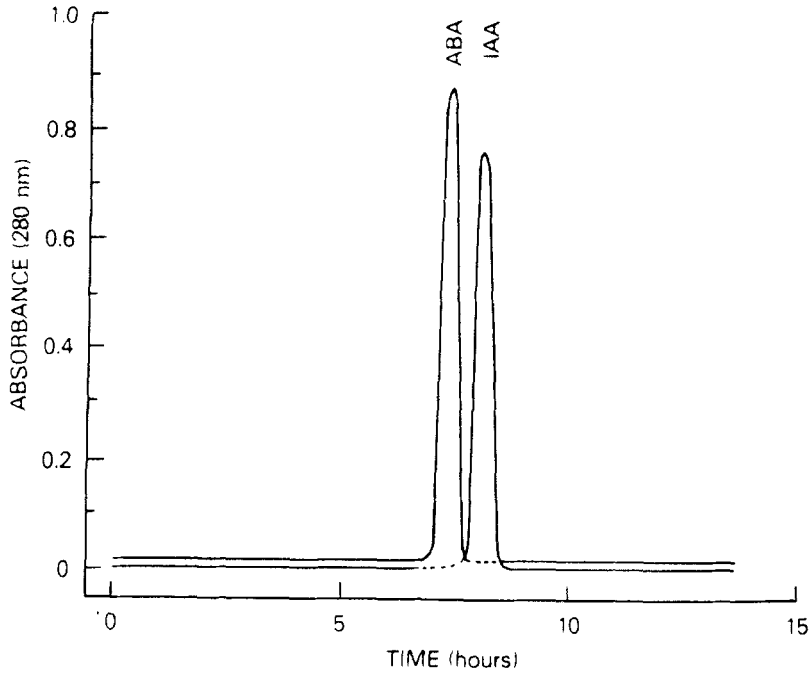

Figure 4. Separation of ABA and IAA by toroidal coil planet centrifuge

which ABA is readily separated from the indoles. Our previous paper (4) also reported the effective separation of gibberellins (GA_3,GA_4 and GA_7) by CCC in ethyl acetate-methanol 0.5M phosphate buffer, p_H 5.9 (3:1:2). Further, we have demonstrated that, for separation of cytokinins (zeatin and 6-isopentenyl adenine and their ribosides), either phase of the solvent system containing ethyl acetate-methanol-0.5M phosphate buffer, p_H 7 (3:1:3) could be used as the stationary phase which is analogous to normal and reversed phases in liquid chromatography.

In the present work, we have first standardized the procedure for separation of ABA with a mixture of ABA and indole-3-

acetic acid (IAA) in chloroform-acetic acid-water (2:2:1) keeping the organic phase stationary (FIG. 4). For ABA analysis from plant tissue, an acidic extract from Zoysia grass seed was subjected to separation by HPLC on a reversed-phase μ Bondapak C_{18} column using methanol-water (80:20) and a fraction corresponding to the retention volume of the standard ABA sample was collected. This fraction (100 μg) in 100 μl of methanol was introduced, via sample port, on to CCC column which contained the stationary organic phase and eluted with aqueous phase. The peak corresponding to the retention time of standard ABA was collected and the solvent evaporated to give a sample residue (22 ng) which was confirmed as ABA by HPLC under the conditions mentioned above (FIG. 5). A portion of the sample was converted into the methyl ester with diazomethane and subjected to GC-MS analysis which showed that the sample from Zoysia grass seed extract is indeed ABA methyl ester (FIG. 6).

2) Preparative CCC. To simplify the clean-up procedure of the crude plant extracts prior to CCC analysis of ABA, we have used preparative CCC which is capable of handling gram quantities. The chromatographic profile obtained from preparative CCC run for a standard mixture of ABA (100 mg) and IAA (100 mg) utilized a solvent system composed of 1600 ml chloroform, 1600 ml acetic acid and 800 ml water. The chromatograph was operated at 45 rpm and the column was filled with aqueous (upper) phase and eluted with organic (lower) phase maintaining the flow rate at 3.1 ml/min (UV detection at 260 nm). After standardizing the chromatographic operating conditions (retention times at a given flow rate for eluting solvent and revolutional speed) for standard ABA and IAA mixture (FIG. 7), the system was applied for plant sample analysis. The sample from plant extract (see Materials and Methods) (up to 1 g sample in 1 ml methanol) was introduced via sample port (a Rheodyne injector was attached to the instrument for sample introduction). By applying the above flow rate

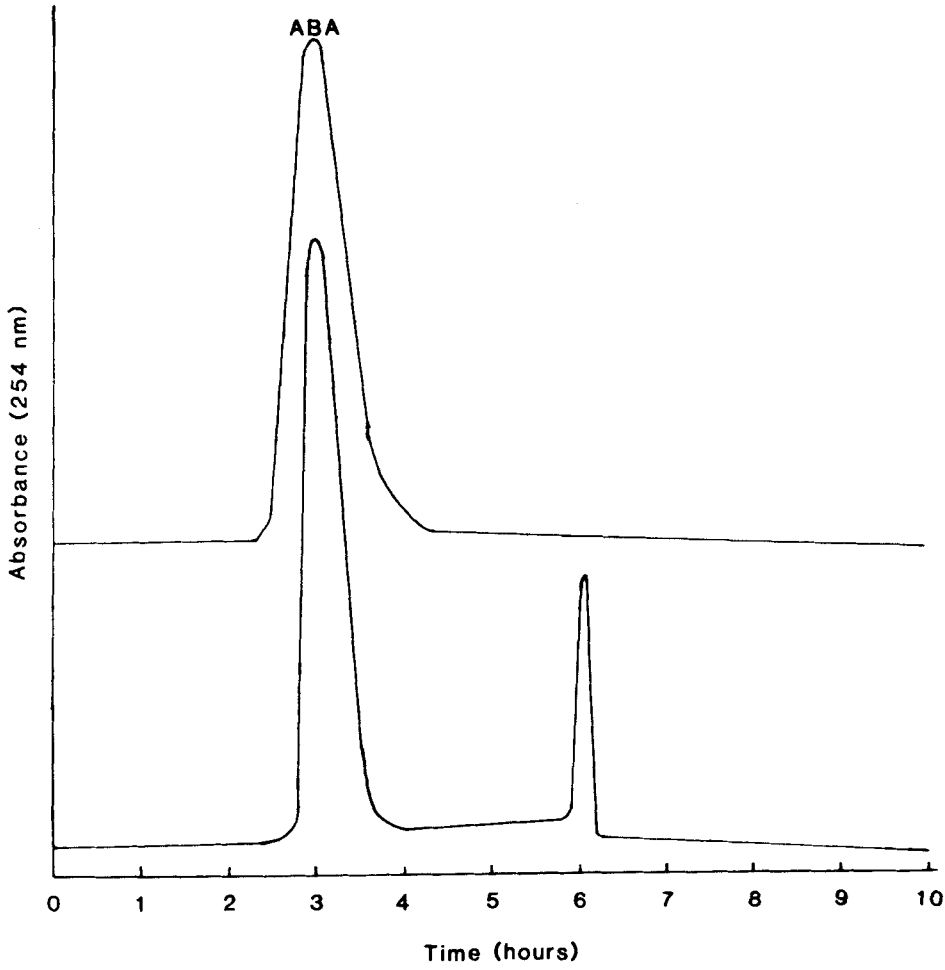

Figure 5. HPLC profile of ABA from Zoysia grass seed after CCC separation

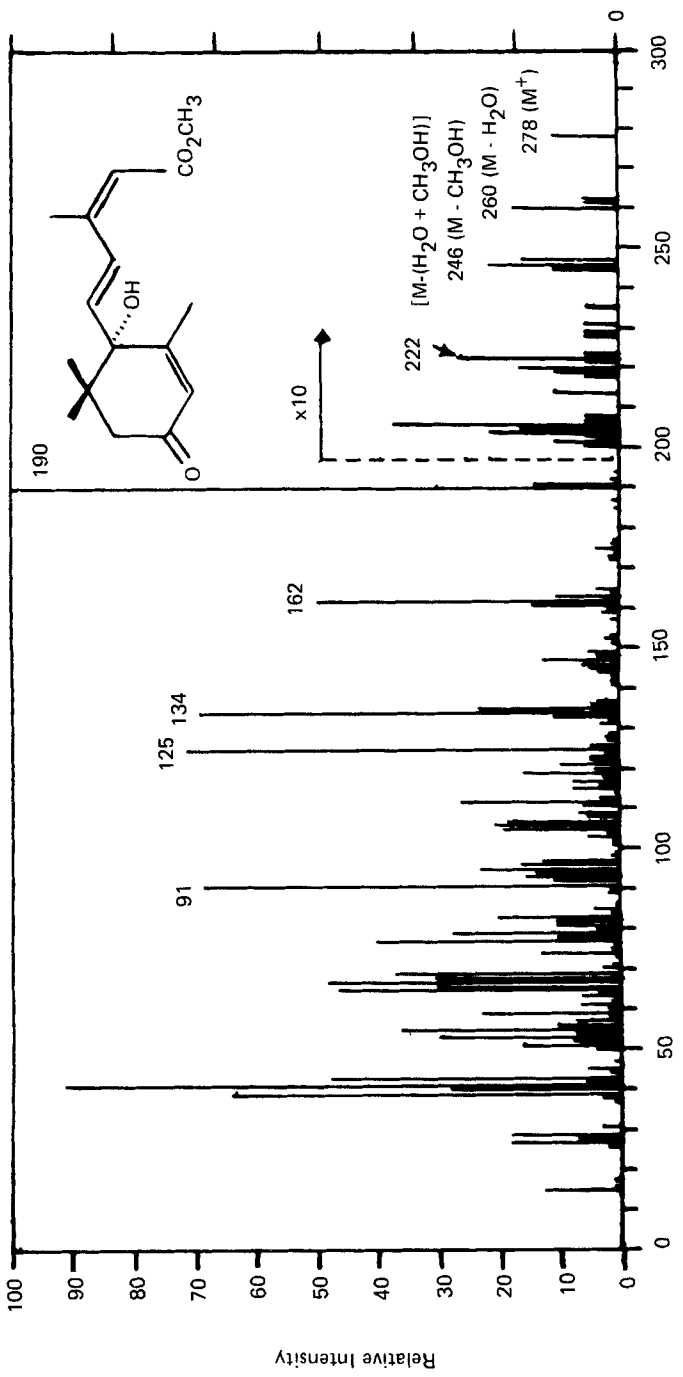

Figure 6. MS of ABA-methyl ester obtained from CCC separation of ABA

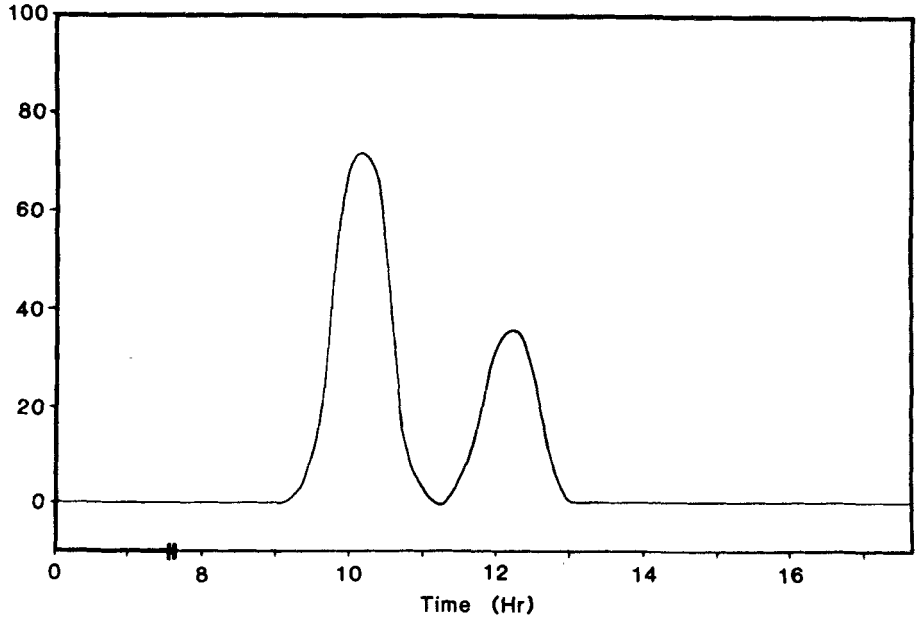

Figure 7. Separation of ABA and IAA by preparative CCC

under the optimum revolutional speed of 50 rpm, the eluate was monitored at 260 nm with a UV detector and the fraction corresponding to the retention time of standard ABA was collected, thus separating it from other undesired compounds. To insure that this fraction contained ABA, it was qualitatively checked for spectrum by an ultraviolet spectrophotometer. This fraction after evaporation of the solvent was subjected to toroidal CPC which separated ABA from other contaminants. Final confirmation for ABA from the sample (purified in this manner) was obtained by GC-MS analysis of the corresponding methyl-ester. The two-step procedure (preparative CCC followed by analytical CCC) eliminated the need for time consuming precolumn clean-up work of crude plant extracts.

TABLE 2.

Abscisic Acid Analysis by Countercurrent Chromatography

	Plant sample	ABA content* (ng/g plant tissue)
1.	Zoysia grass seed	22
2.	Soybean seed	26
3.	Avocado fruit	15

* The values reported here are only approximate (estimated error, $\sigma = 4.86$) and based on the average of three experiments.

Although HPLC is generally considered to be faster and more efficient method than CCC, we noticed the following disadvantages in the hormone analysis:

1) As for the sample size limitations, even on preparative HPLC column, the sample loading capacity cannot exceed 200-500 mg of the crude plant extracts.

2) Deterioration of expensive HPLC columns during clean-up with strong solvents sometimes causes the need for replacement of columns and this incurs additional expenses.

Utilizing the 2-step CCC procedure, we have analyzed representative classes of plant tissues (TABLE 2) to demonstrate its potential use for ABA analysis.

3) High-Speed Preparative CCC. While our aforementioned work using analytical and preparative CCC instruments was in progress, another CCC prototype for semi-preparative analysis was reported (7). The main advantage of using this CCC instrument is that it has shorter time duration for analysis as compared to other in-

struments (described above) without sacrificing resolution during separation. It can also be used for both analytical and preparative works because the coiled column assemblies can be changed depending on the size of the sample. In other words, a set of coiled column assemblies contains different sizes of the columns which can handle from micrograms (small column akin to analytical CCC column) to gram quantities (large column). A design similar to this CCC instrument is now commercially available (P.C. Inc., Potomac, MD). To determine its potential for plant hormone analysis, milligram amounts of a mixture of indole compounds were tested for the efficiency in the resolution of peaks during separation into individual components (FIG. 8A). The resolution of this mixture by the toroidal CPC (FIG. 8B) had already been reported. It is clear from Figure 8 that this instrument is far more superior than the other CCC instruments because of the differences in compound separation times and loading capacity of the sample. To further demonstrate its utility for ABA analysis, we have separated a mixture containing 50 mg ABA and 50 mg IAA. The separation was completed within 2 hours and the solvent required for CCC run was about 200 ml (FIG. 9). This is comparable to a typical HPLC run which takes about 2 hr and requires about 200 ml solvent. The potential use for this instrument appears to be great because of the shorter times (thereby quick runs) and lesser amount of solvent as compared to the preparative CCC.

This work suggests the possibility for an alternative analytical method in the separation science, particularly for trace analysis such as hormone analysis from plant tissue. At the present stage of the development, the CCC method supplements (complements) HPLC. The main advantage in CCC is that the column has no solid support thereby eliminating such problems as sample adsorption, deterioration (loss) of sensitive/ labile samples, besides the expenses involved for column purchases, which are

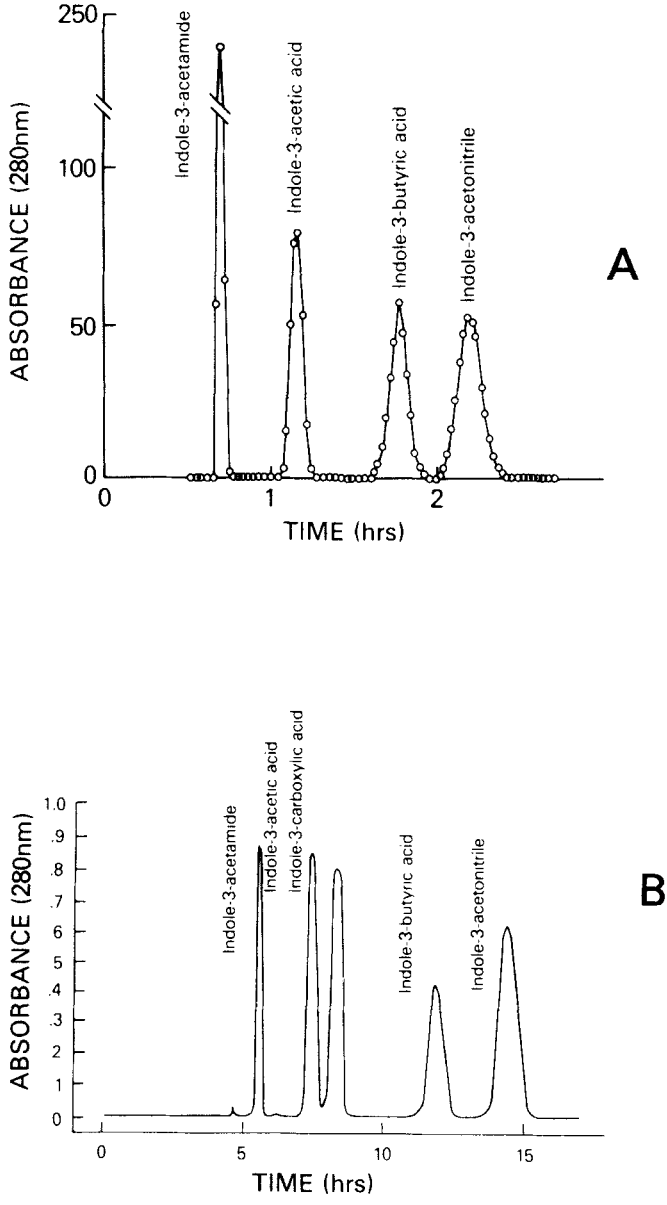

Figure 8. Separations of indole hormones by semi-preparative (A) and analytical (B) CCC. A: Chromatogram obtained by high-speed CCC; B: Chromatogram obtained by Toroidal CPC

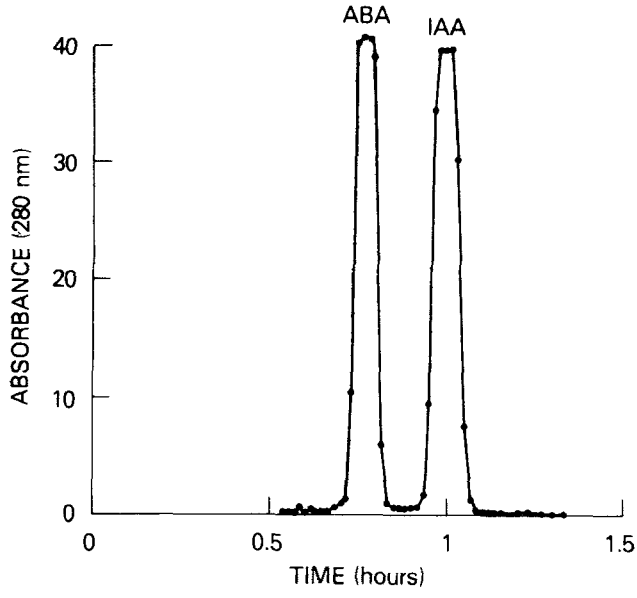

Figure 9. Separation of ABA and IAA by high-speed semi-preparative CCC

sometimes encountered in liquid chromatography. Also, there is no need to get different types of normal and reversedphase columns (as in HPLC) because the mobile and the stationary phase solvents can be interchanged and the column clean-up as well as the sample recovery are easily accomplished. For many researchers working with limited funds, cost for the purchase of the instrument is of a great concern and the CCC instruments offer alternative source because they are available under $10,000. In other words, this type of separation method appears to be an obvious choice when one considers the price and advantage of not using solid support in the column as long as the CCC and HPLC separations are comparable in the separation and analysis of organic compounds.

DISCLAIMER

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